Enhanced Fluidity Liquid Chromatography for Biological Applications

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Abstract

The following work describes the successful application of enhanced fluidity liquid (EFL) mobile phases to improving isocratic chromatographic separation of biological molecules in hydrophilic interaction liquid chromatography (HILIC) mode. The mobile phase was buffered methanol/water with carbon dioxide added to create the enhanced fluidity liquid. Nucleosides (adenosine, uridine, cytidine, guanosine) were employed as the test sample for this method comparison. Using UV detection at 262 nm, the separation of the sample molecules was studied under each mobile phase condition. Increases in peak resolution between all four were observed as a function of increasing additions of carbon dioxide to create the enhanced fluidity liquid. This increase in resolution was achieved by a combination of improvements in method selectivity and separation efficiency. Plate height was seen to decrease by up to 27%, retention increased for all compounds, and the separation factor for the originally co-eluted cytidine-uridine peak pair increased from 1.03 to 1.24. This resulted in the final baseline resolution of cytidine and uridine. This study marks the first report of EFL being used in conjunction with HILIC.

Background

Chromatography in its most general sense describes the separation of molecules by differences in equilibrium between two distinct phases, one of which is mobile. The movement of one phase, the mobile phase, relative to the other phase, the stationary phase, allows for the separation of molecules on the chromatographic device, leading to temporal differences in sample molecules exiting the chromatographic device. Here and in all future discussion within this section, discussion will be limited to liquid chromatography (LC) which is characterized by a liquid mobile phase. The stationary phase for LC is typically functionalized particles packed into column. [1]

Retention on this column is related to the retention factor, k, for a specific molecule in a chromatographic system. Thermodynamically, the degree to which a compound is retained on the column is related to its equilibrium constant K for the stationary and liquid phases and the volumes of the stationary and mobile phases, V_s and V_m respectively and is expressed in Eqn 1. [2]

$$k = K\frac{V_s}{V_m} \quad (1)$$

Chromatography allows for retention factor to be easily calculated by using the time required for an unretained compound to leave the column, t_m, and the retention time, t_r, which is the time required for the molecule of interest to leave the column. This allows for a more convenient calculation of k, as shown in Eqn. 2. [3]

$$k = \frac{t_r - t_m}{t_m} \quad (2)$$

It is the difference in retention times, controlled by the difference in retention factors, that provides a separation of two molecules in LC. The relative difference (Eqn. 3) in retention factors is known as the selectivity factor, α. [3]

$$\alpha = \frac{k_2}{k_1} \tag{3}$$

This relation can also be written as the ratio of the equilibrium constants for the two analytes in the chromatographic system. [2] The ratio of equilibrium constants simplifies to Eqn. 4, showing that thermodynamically α represents the difference in Gibbs free energy for the partition for each molecule between the two phases; α can be written in terms of the difference in the Gibbs free energy, ΔΔG, the gas constant R, and the absolute temperature T, as shown in Eqn. 4.

$$\alpha = e^{\frac{-\Delta\Delta G}{RT}} \tag{4}$$

By nature, however, chromatographic separations embody a non-equilibrium facet. As a consequence of the mobile phase moving, molecules are not in a constant state of equilibrium. The non-equilibrium nature of this process makes the bands of sample traveling down the column broaden, creating difficulties in separating closely spaced peaks. This effect is measured in the number of theoretical plates found in a column, N. Each theoretical plate corresponds to one equilibration between the mobile and stationary phases. A greater number of theoretical plates will correspond to greater separation efficiency and narrower sample bands. It is helpful to consider another parameter, plate height, H, which is related to N and the column length L by Eqn. 5. [3]

$$H = \frac{L}{N} \tag{5}$$

Using H allows a more fundamental understanding of the chromatographic process since it is independent of the column length. H can be modeled by the Van Deemter equation, Eqn. 6, in which D_m is the diffusion coefficient in the mobile phases, d_p is the diameter of the packing, u is the linear velocity of the mobile phase, and A, B, and C are Van Deemter coefficients. [4]

$$H = A \cdot d_p + \frac{B \cdot D_m}{u} + \frac{C \cdot d_p^2 \cdot u}{D_m} \quad (6)$$

The A term describes band broadening by way of multiple flow paths through the packed column, the B term describes band broadening through longitudinal diffusion down the column, and the C term describes the impact of resistance to mass transfer on band broadening. It can be seen from Eqn. 6 that the comparative impact of the B and C terms on the efficiency of the separation are dependent on mobile phase linear velocity. Using Eqn. 5, it is shown that reducing H will increase N, leading to a more efficient separation. [2]

Fundamentally, the mobile phase flow profile contribution to the C term, C_m, can be roughly modeled by Eqn. 7, where w is the packing factor and all other parameters previously defined [1]

$$C_m = \frac{w \cdot (1+6k+11k^2)}{96(1+k^2)} \quad (7)$$

This relation reflects the fact that the longer that a chromatographic band stays on the column, the more broadening as a result of flow profile will occur.

The equilibrium and non-equilibrium facets of liquid chromatography combine to give resolution between molecules. The resolution, R, between two molecules is given by Eqn. 8, with all variables having been previously defined. [3]

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{k+1}\right) \quad (8)$$

Resolution can be improved by changing any of the three parameters k, N, or α. By optimizing all three of the parameters, optimal resolution can be achieved for a sample.

Introduction

Liquid chromatography (LC) is an important separation technique for a wide range of biological molecules. Historically, however, limitations were encountered with highly polar molecules that often required pre-column derivitization for effective separation [5,6]. With the introduction of hydrophilic interaction liquid chromatography (HILIC) in the early 1990s by Alpert [7], LC has become much more effective at separating polar molecules, as are often encountered in biological matrices. HILIC employs a hydrophilic stationary phase with a polar organic mobile phase with aqueous additive. This is postulated to create a water-rich layer on the surface of the stationary phase and separation is achieved by the equilibrium of the sample molecules between this water-rich layer and the predominantly organic bulk mobile phase [8]. HILIC is especially ideally suited to separating nonvolatile polar organic molecules such as amino acids [9], peptides[10], organic acids [11], and nucleosides[12]. Since many of these compounds are of prime importance in pharmaceutical applications [13], there is a great deal of interest in improving this separation technique.

A novel method for improving liquid chromatographic separations is the use of enhanced fluidity liquids (EFLs) as the mobile phase. EFLs are traditional LC mobile phases with low-viscosity liquefied gasses added, often employing carbon dioxide [14]. The diffusion coefficient of EFLs has been shown to increase as a function of increasing additions of carbon dioxide [15]. These phases, often comprised of methanol-CO_2-water, are kept in phase by pressurizing the chromatographic system [16]. Work has been done to determine the phase diagrams for EFLs created with methanol to ensure that adequate experimental parameters can be maintained [17].

The advantage of employing an EFL for a chromatographic separation is that it has a higher diffusion coefficient than traditional liquid mobile phases. This is important since plate

height is inversely proportional to the diffusion coefficient in the mobile phase [4], as shown in Eqn. 9, simplified from Eqn. 6, where H is plate height, D_m is the diffusion coefficient in the mobile phase, d_p is the particle diameter, u is the linear velocity of the mobile phase, and C is the Van Deemter coefficient for resistance to mass transfer.

$$H \propto \frac{C \cdot d_p^2 \cdot u}{D_m} \tag{9}$$

This relation holds true since LC is generally run at sufficiently high flow rates that the C term dominates since it is directly proportional to mobile phase linear velocity. Eqn. 8 shows that by increasing the diffusion coefficient in the mobile phase, the overall plate height for the separation should decrease. The EFL approach to increasing the diffusion coefficient has been shown to give similar results to the more broadly applied method of raising the column temperature [18]. Increasing the diffusion coefficient by using an EFL, however, has the marked advantage of avoiding elevated temperatures, which can degrade heat-sensitive biological molecules.

EFLs have been shown to increase separation efficiency for reversed-phase separations [18,19], normal-phase separations [20], and chiral separations [21]. There has been no published work applying EFLs to HILIC separations, however. The interest in employing a CO_2-based EFL in HILIC is that in addition to the diffusion increases that are commonly seen with the use of an EFL, the liquefied gas also impacts the polarity of the mobile phase. While mobile phase polarity change occurs in any EFL, this has not been explored in the previous investigations since they employed relatively nonpolar mobile phases. The polar organic-aqueous mobile phase employed in HILIC, however, will show a marked change in solvent strength with the addition of non-polar CO_2.

This change in solvent strength will affect k for the separation, with a requisite impact on the efficiency of the separation as shown in Eqn. 7. This effect will lead to an increase in H as a function of added CO_2 since non-polar CO_2 will increase k. Part of the interest in investigating HILIC using an EFL is to investigate whether this increase in H as a function of increasing k will be offset by the decrease in H as a function of increasing D_m, both of which occur as a function of increasing CO_2 addition to the mobile phase.

Additionally, using a CO_2 –based EFL has practical benefits in terms of cost of analysis and environmental impact of the separation. CO_2 can be purchased at a fraction of the cost of more common HILIC additives such as acetonitrile. By effectively displacing some of the organic in the mobile phase, the cost of analyzing a sample decreases. Additionally, CO_2 is environmentally neutral, requiring no specialized disposal protocol. This is again a marked improvement over other organic solvent, which often require significant effort and specialized handling to be disposed of.

The compounds studied in this investigation were the nucleosides adenosine, uridine, cytidine, and guanosine shown in Figure 1. These compounds are of interest to pharmaceutical chemistry since nucleoside levels can be used in metabolomics as disease markers [22,23], and in food chemistry for product analysis and identification [24]. Since such analyses are often done in complex biological matices, efficient, selective separation techniques are required for analysis. Current techniques for analyzing nucleosides are electrophoresis [22] and HPLC [23,24], but both techniques require extended analysis times, often taking over 40 minutes in HPLC mode. Additionally, almost all current HPLC techniques require the use of a gradient to obtain a desired separation. Gradients can be complex to execute in practice and long run times result in lower sample throughput for a lab and longer individual sample analysis times. Finally, most HPLC

techniques rely upon the use of acetonitrile-based mobile phases and gradients, and this can be an issue from a cost and environmental prospective, as mentioned above. This investigation centered on the separation of nucleosides using a HILIC column and methanol/water LC mobile phase modified with CO_2 to yield an EFL. This approach utilizes the advantages of EFL to improve the efficiency and selectivity of the separation. This investigation took a particular look at the equilibrium and non-equilibrium aspects of the separation to give a complete understanding of the resolution process. Additionally, this method gives the added advantage of being investigated entirely in isocratic flow, which is often easier to work with than gradient methods.

Experimental

HPLC Setup

The HPLC system was assembled in the lab from commercially available components. The pump was an ISCO 260 D syringe LC pump (Teledyne Isco, Inc. Lincoln, Nebraska, USA). The pump was connected by 0.02 inch stainless steel tubing (VICI Valco Instruments, Houston, Texas, USA) to a Valco 6-port 5000 psi injector equipped with a 2 µL injection loop (VICI Valco Instruments, Houston, Texas, USA). The injector was then connected to the HPLC column. This investigation employed a 4.6x150mm Tosoh Amide-80 column packed with 3 µm particles (TOSOH Bioscience, King of Prussia, Pennsylvania, USA). This column was selected on the basis of its amide functionality, which is resistant to acid attack. This is important since the CO_2 modifier can react with the water in the LC mobile phase to create carbonic acid.The column was then connected to a Jasco UV-2075 UV-Vis detector with a high-pressure 4 µL flowcell (Jasco Inc. Easton, Maryland, USA). The outlet of the flowcell was equipped with a fused silica capillary with 30 µm internal diameter and 365 µm outer diameter (Polymicro Technologies, Inc. Phoenix, AZ, USA) of a certain length to maintain system pressure.

LC Preparation

The LC mobile phase was consistently created as 90/10 v/v methanol/water. The methanol (Fisher Scientific, Pittsburgh, Pennsylvania, USA) was spectroscopy grade and the deionized water was purified on a Barnstead Nanopure Infinity system (Thermo Scientific, Asheville, North Carolina, USA). The water was buffered with sodium acetate (Jenneile Enterprises, Cincinnati, Ohio, USA) and acetic acid (Mallinckrodt Baker, Inc. Phillipsburg, New Jersey, USA) to a buffer strength of 20 mM and an aqueous pH of 4.4. This buffer was introduced to

counteract acid formation by the interaction of CO_2 and water. The buffer was stored at 4ºC when not in use to enhance its stability. The buffer was filtered before making the mobile phase to ensure that residual particulates did not enter the chromatographic system. The final methanol/aqueous solution was degassed for 20 min before use using a Branson 2210 ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, Connecticut, USA). The degassed liquid solution was then transferred to the syringe pump for use.

EFLC Preparation

The buffered methanol/water LC mobile phase described above was held in the HPLC syringe pump and pressurized to 80 bar. In a second syringe pump, chromatography grade liquid carbon dioxide (Praxair, Inc. Danbury, Connecticut, USA) was held at 80 bar. The volume of LC mobile phase was recorded and then with all valves closed on the HPLC pump, then a free space was opened in the HPLC pump by running the pump down, creating a vacuum over the LC mobile phase. The volume of this headspace was monitored to ensure that there was more than enough room for the prescribed volume of CO_2 to enter without hindrance. The carbon dioxide was then added by stainless steel tubing linking the two pumps together. Once the carbon dioxide was added, the syringe pump on the HPLC system was then cycled up and down to assist in the proper mixing of the two phases. The HPLC was then pressurized to 200 bar overnight to allow complete equilibration between the LC phase and the CO_2 to create a homogenous EFL. Throughout this document, the EFL phases are referred to by the volume percent of CO_2 that was added to the LC phase to create the EFL. EFLs can also be characterized terms of mole fraction of CO_2 in the final EFL, which has been used to characterize EFL mobile phases in the past [15].

For the phases prepared in this investigation, the 20 volume percent CO_2 added EFL is 0.11 mole fraction CO_2 and the 40 volume percent volume added EFL is 0.20 mole fraction CO_2. [25,26]

Sample Preparation

All samples were prepared in LC mobile phase. All samples were created at a concentration of 10µg/mL. 99% uridine, 99% adenosine, 99% cytidine, and 98% guanosine (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) were used to create the samples. Stock solutions of each nucleoside were prepared separately at 0.2 mg/mL and held below -10 ºC. Test samples and mixes were prepared from these stock samples in LC mobile phase at a concentration of 10µg/mL as needed. While running in LC mode, a spike of toluene was used to mark column dead time. All samples were filtered through a 0.45 µm filter before use and kept below -10 ºC while not in use.

Data Analysis

Chromatographic data were recorded using EZ Chrom Version 6.7 (Scientific Software Inc. Pleasanton, California, USA). Data analysis was performed using PeakFit Version 4 (SPSS Inc. Chicago, Illinois, USA). All peak values were used as displayed from PeakFit. Efficiency calculations were calculated by Peakfit using moment analysis and retention times were found as the top of each peak. Peak fitting calculations were iterated until there was no increase in goodness of fit perceived from increasing fitting calculation iterations. All efficiency and retention factor studies were done by analyzing fully resolved or single peaks to ensure that accurate values for k and N could be calculated. Four-nucleoside mixtures were only used to create sample chromatograms and to determine peak resolution using statistical moment analysis.

Method

Since the objective of the investigation is to compare HILIC separations under LC and EFL modes, all parameters and equipment were unchanged except for the mobile phase and the flow restrictor for the EFL solutions. When the HPLC was run in LC and EFL modes, it was run in constant flow mode to ensure constant mobile phase linear velocity. While using EFLs, however, care was taken to ensure that sufficient pressure was maintained to keep the EFL in phase throughout the separation. The injector and injection needle were thoroughly cleaned in between samples to ensure minimal injector carryover between injections of different samples.

All efficiency and retention data were taken in triplicate to ensure the reproducibility of the data. The efficiency and retention data were taken at a variety of flow rates to see the dependence of H on mobile phase velocity. Selectivity, resolution, and retention data were taken for LC conditions and 20 and 40 volume percent CO_2 added EFLs. Efficiency data were recorded for the LC and 20 volume percent CO_2 added EFL to give an initial look at the effect of EFL on efficiency, since efficiency data acquisition required sampling a large number of flow rates to determine the linear velocity dependence of H. All of this work was done at sufficiently high flow rate as to have H primarily dependent on the resistance to mass transfer of the sample between the mobile and stationary phases. When using EFLs, the column was allowed to equilibrate to the new mobile phase before any chromatography was done. After using an EFL mobile phase, the column was flushed with LC mobile phase for overnight storage. This was in an attempt to mitigate column damage from the CO_2 coming out of solution and remaining in the column for long periods of time during overnight storage.

Results

Effects on Retention

The investigation into the effect of EFL use on compound retention was studied using the LC mobile phase and comparing it to EFLs created from the addition of 20 and 40 volume percent of CO_2. As seen in Figure 2, the retention of all the compounds increased as a function of volume percent of CO_2 added to create the EFL. All work was done at 0.4 mL/min in triplicate, but the 95 percent confidence intervals for the replicates are smaller than the data points in Figure 2. The following comparisons are between the LC condition and the 40 volume percent CO_2 added EFL. Adenosine showed a 72% increase in retention with a final k of 0.93. Uridine showed a 66% increase in retention with a final k of 1.33. Cytidine showed a 97% increase in retention with a final k of 1.64. Guanosine showed a 138% increase in retention with a final k of 2.5. From these data it can be seen that the k values with the use of a 40 volume percent carbon dioxide added EFL approach the optimal k values of 2-10 [2]. The advantage of increasing k above 2 is that weakly retained compounds are very susceptible to band broadening by extra-column effects. This increased in retention was the expected result since in this separation the non-polar carbon dioxide is functioning as a weak solvent, which should increase retention of the sample molecules.

Effects on Separation Factor.

Using the data collected for the retention factor, α for neighboring peaks was also calculated. Figure 3 shows the changes in α as a function of increasing CO_2 addition to the mobile phase. The following discussion compares the value of α between the LC condition and the use of a 40 volume percent CO_2 added EFL. The adenosine-uridine peak pair showed an increase in α from

1.23 to 1.42. The uridine-cytidine peak pair showed an increase in α from 1.03 to 1.24. The cytidine-guanosine peak pair showed a decrease in α from 1.73 to 1.52. This decrease in α was not detrimental to the separation since there was still ample selectivity between cytidine and guanosine under both EFL conditions. These changes in α corresponds with the observation that the retention of the sample molecules increased at differing rates, allowing the separation factor to generally increase as a function of added carbon dioxide.

Effects on Efficiency

The efficiency study, involved the comparison of buffered 90/10 v/v methanol/aqueous liquid mobile phase and an EFL prepared by adding 20 volume percent of carbon dioxide to the liquid mobile phase. It was found that enhanced fluidity liquids affected the separation efficiency of the nucleosides. As seen in Figure 4, for weakly retained adenosine the efficiency increased with the use of an EFL. This compound showed an approximately 25% decrease in H for all linear velocities sampled. As seen in Figure 5, however, the next most retained molecule, uridine, had negligible change in separation efficiency under EFL conditions as compared with traditional LC conditions. Figures 6-7 however show that both cytidine and guanosine experienced increases in plate height under EFL conditions. This was not surprising since they were the last two eluting molecules and had the proportionately greatest increases in k with the addition of CO_2.

Effects on Resolution

When all four compounds were introduced onto the column together for separation, the resolution between the compounds was measured. As seen in Figure 8, the resolution between all of the peak pairs increased as a function of increasing fraction of carbon dioxide in the EFL

mobile phase. For the adenosine-uridine pair, resolution increased by over 187% between the LC condition and the use of a 40 vol% CO_2 addition EFL, giving a final resolution of over 3. For the uridine-cytidine pair, originally co-eluted under LC conditions, a resolution of nearly 2 was achieved using a 40 vol% CO_2 added EFL. For the cytidine-guanosine pair, resolution increased by over 22% between the LC condition and the use of a 40 vol% CO_2 added EFL, giving a final resolution of over 3.75. All of the final resolutions that were achieved sufficiently baseline resolve the components of this mixture. This increase in resolution can also be visualized in Figures 9-11, which depict the chromatograms that the resolution data were computed from. It can be seen from these chromatograms that all peaks are fully baseline resolved with the use of a 40 vol% CO_2 added EFL.

Discussion

The advantages seen by using EFLs as mobile phases for HILIC separations have been shown to be from a combination of decreases in plate height and increases in selectivity for the given molecules. The increases in α seen in the separation had the greatest influence on the increase in resolution seen while using the 40 volume percent CO_2 added EFL. This was especially true for the initially co-eluted cytidine-uridine pair.

The interesting aspect of this investigation, however, was impact on H with the use of EFLs. As was stated in the introduction, H is increased with an increasing k while it is inversely proportional to D_m. Since and EFL increases both of these parameters, their interaction is critical for understanding the applicability of this method. Combining equations 8 and 9, it can be seen that the change in H, as a function of change in D_m and k, can be approximated by Eqn. 10 where f(k) is Eqn. 9.

$$\Delta H \propto \frac{\Delta f(k)}{\Delta D_m} \tag{10}$$

Table 1 shows the comparison of the experimental data to the expected result modeled by Eqn. 10. All changes are shown as relative values to the LC condition to account for the fact that this comparison is using a proportionality to model the entire system. Studies have been done to calculate the D_m for methanol-CO_2 EFLs [15] and this work is the basis for the estimation of the relative change in D_m with the use of an EFL. The k values for the LC condition and the 20 volume percent CO_2 added EFL were used without modification. The values of ΔH were computed for all 5 flow rates and averaged to give the final experimental value of ΔH.

Table 1 shows that the experimental data qualitatively tracks with theory. Based on the diffusion increase alone under EFL conditions, it would be expected that H would decrease by approximately 30%. This decrease was largely seen in adenosine but not in the other

components. The more strongly retained compounds than adenosine saw less decrease in H and even increases in H. This was due to the impact of k on H. Uridine, guanosine, and cytidine were expected to have increasingly smaller decreases in H than adenosine since they had larger Δk values. This trend was seen experimentally, and the relative change in k was shown to accurately make qualitative predictions about relative changes in H. This shows that when using an EFL in a HILIC system, the increases in both D_m and k will have significant impact on the separation efficiency and must both be taken into consideration.

This impact on k is an additional positive attribute to CO_2-modified HILIC EFLs. CO_2 is relatively non-polar, having a Hildebrand solubility parameter of 8.9 [27]. The ability of this relatively non-polar solvent to be freely miscible with a very polar methanol/water mobile phase is a unique characteristic of using a liquefied gas in an EFL. Similar modification would be more difficult using organic solvents of similar polarity to CO_2.

In terms of resolution, it was shown through this analysis that good resolution could be achieved using an isocratic EFL method. This represents two positive attributes of using an EFL to do a HILIC separation. First, the use of an isocratic method allows for simpler analysis than use of a gradient, as is often down for a HILIC separation. Additionally, such good resolution was achieved using the EFL that this separation could be accomplished much faster than was done for this preliminary study and still retain adequate resolution for the method to be of analytical relevance. The combination of these two attributes shows this method to be a fast, straightforward method of doing biological separations of nucleotides in HILIC mode.

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Figures

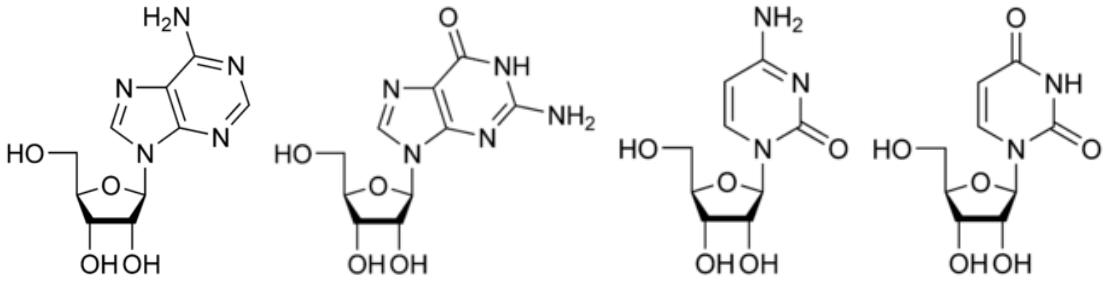

Figure 1. RNA nucleosides used in the investigation

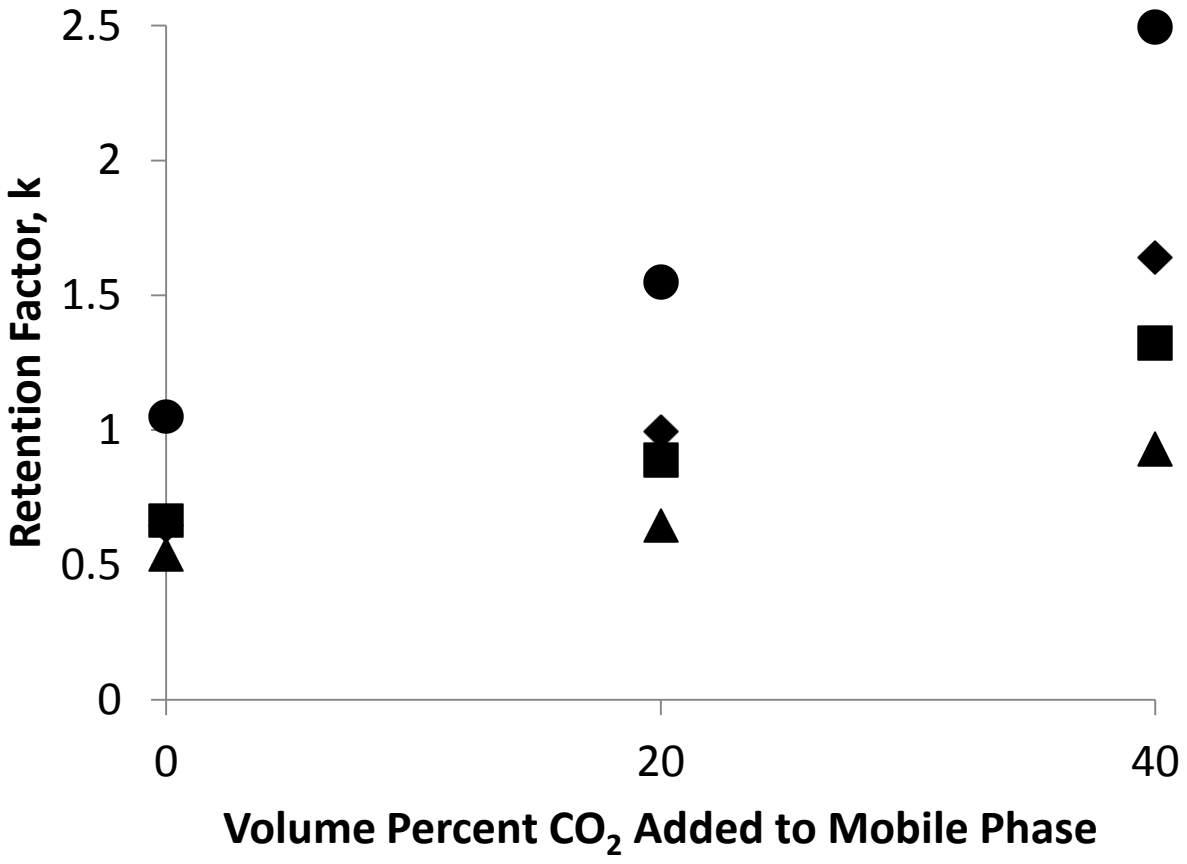

Figure 2. Variation of retention factor as a function of mobile phase composition. All data taken at flowrate of 0.4 mL/min. n=3. ▲ adenosine ■ uridine ♦ cytidine • guanosine

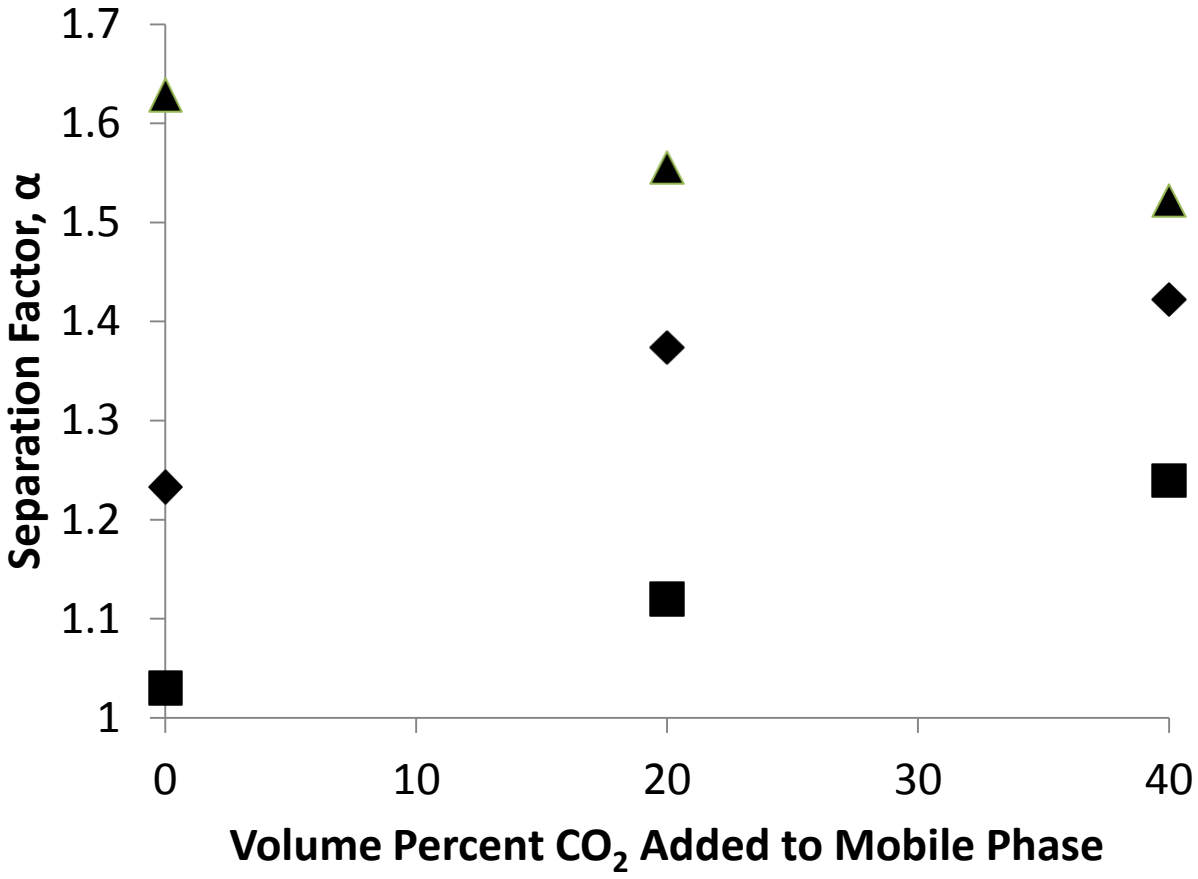

Figure 3. Variation of separation factor (α) as a function of mobile phase composition. All data taken at flowrate of 0.4 mL/min. n=3. ♦ adenosine-uridine ■ uridine-cytidine ▲ cytidine-guanosine

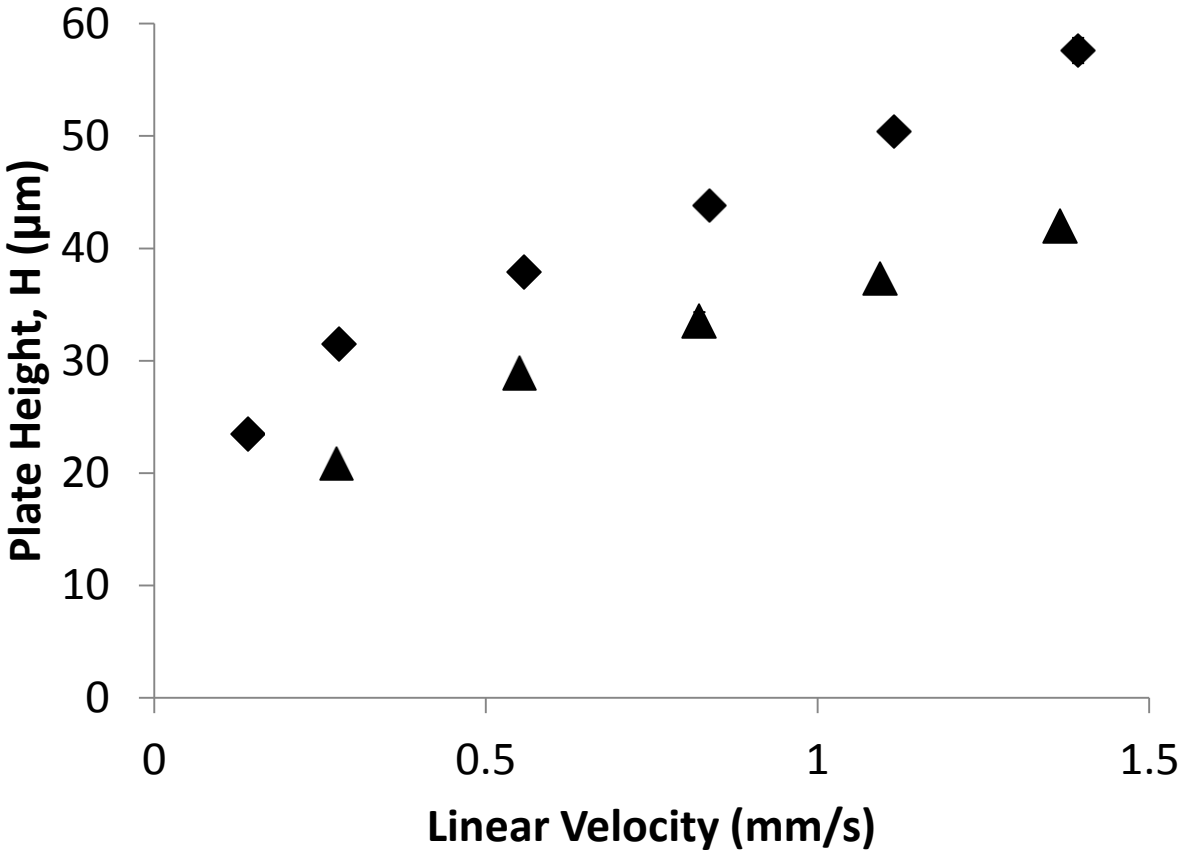

Figure 4. Effect of mobile phase composition on separation efficiency as a function of linear velocity. Analyte is 10 µg/mL adenosine. n=3 for all points, error bars are 95% confidence intervals. ♦ LC condition ▲20 vol% EFL

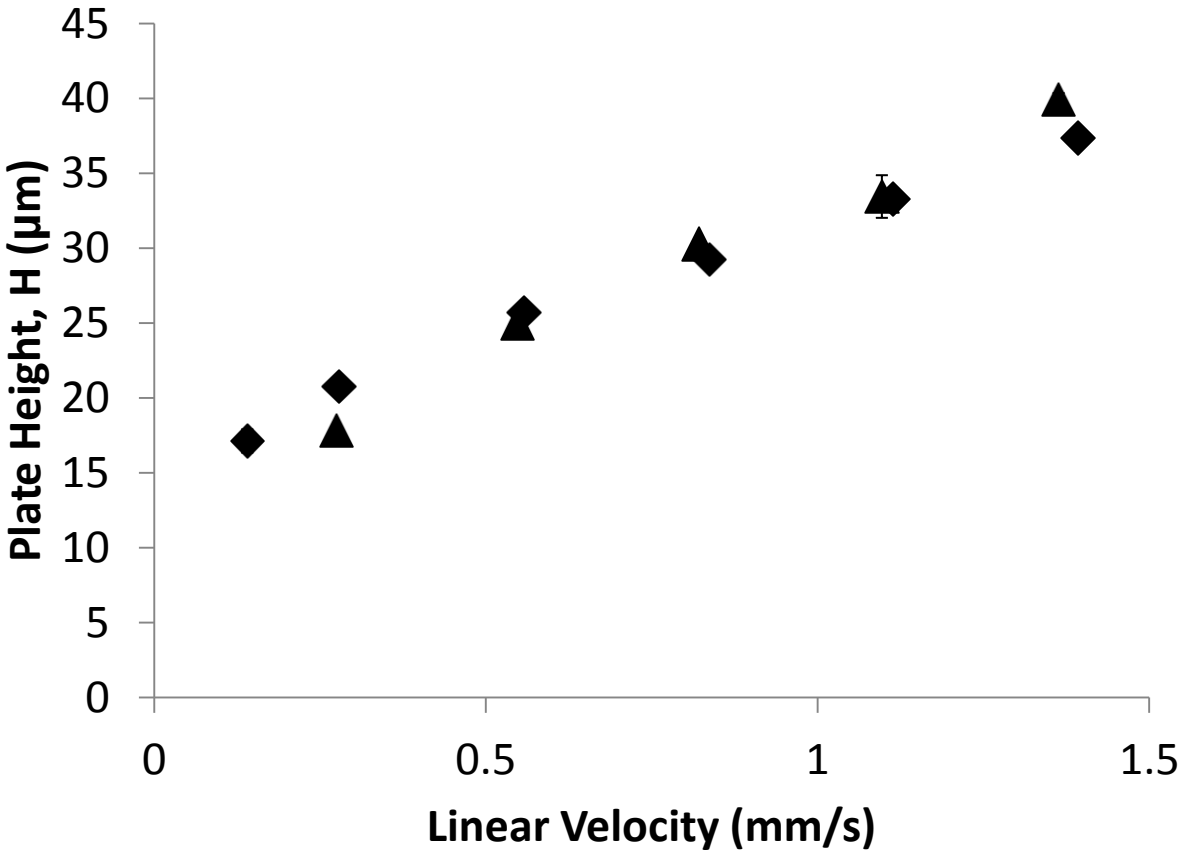

Figure 5. Effect of mobile phase composition on separation efficiency as a function of linear velocity. Analyte is 10 µg/mL uridine. n=3 for all points, error bars are 95% confidence intervals. ♦ LC condition ▲20 vol% EFL

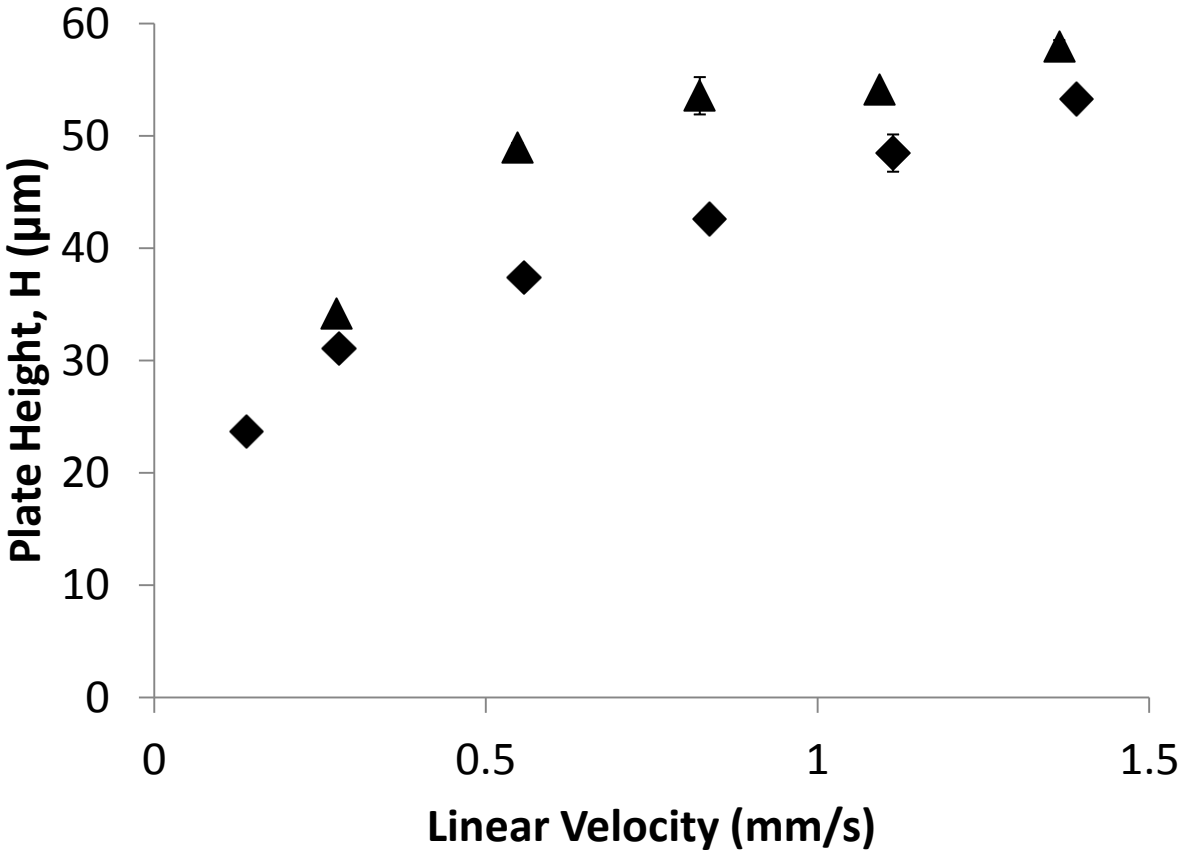

Figure 6. Effect of mobile phase composition on separation efficiency as a function of linear velocity. Analyte is 10 µg/mL cytidine. n=3 for all points, error bars are 95% confidence intervals. ♦ LC condition ▲20 vol% EFL

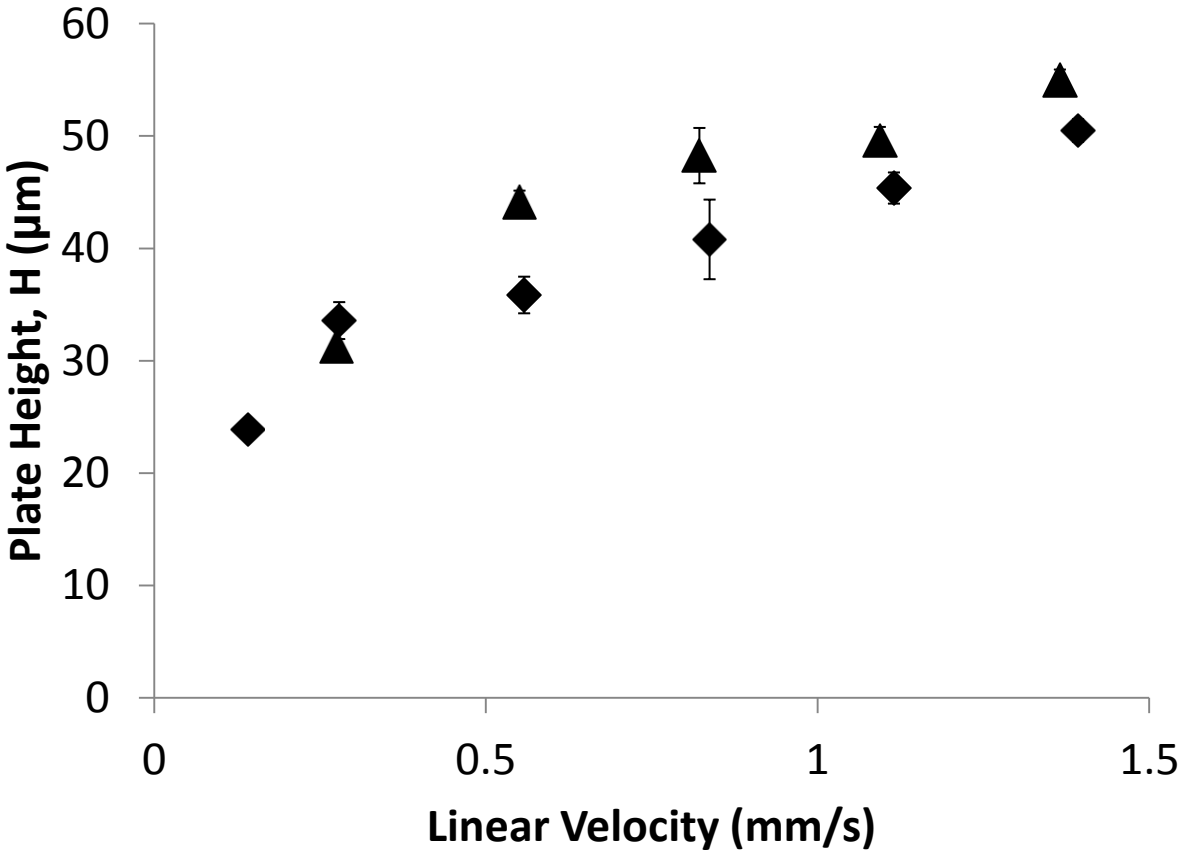

Figure 7. Effect of mobile phase composition on separation efficiency as a function of linear velocity. Analyte is 10 µg/mL guanosine. n=3 for all points, error bars are 95% confidence intervals. ♦ LC condition ▲20 vol% EFL

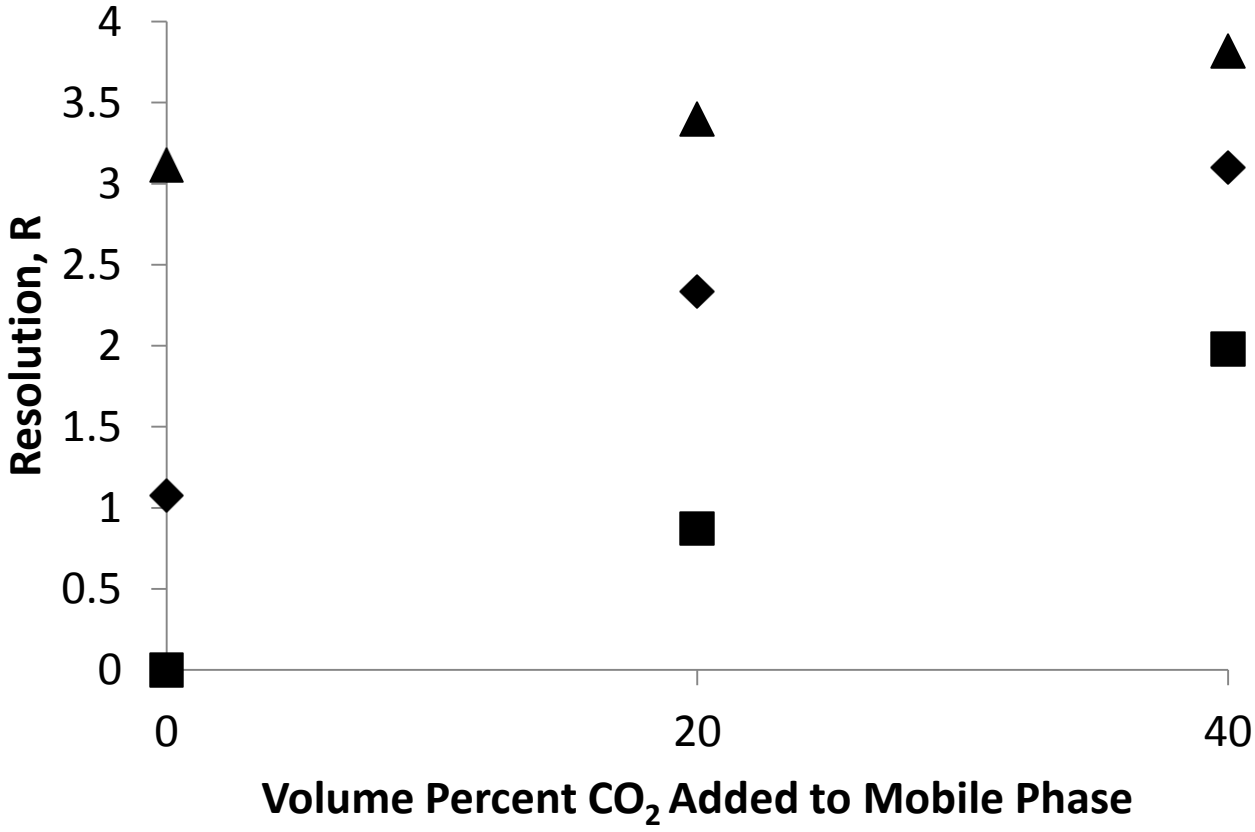

Figure 8. Variation of resolution as a function of mobile phase composition. All data taken at flowrate of 0.4 mL/min. ♦ adenosine-uridine ■ uridine-cytidine ▲ cytidine-guanosine

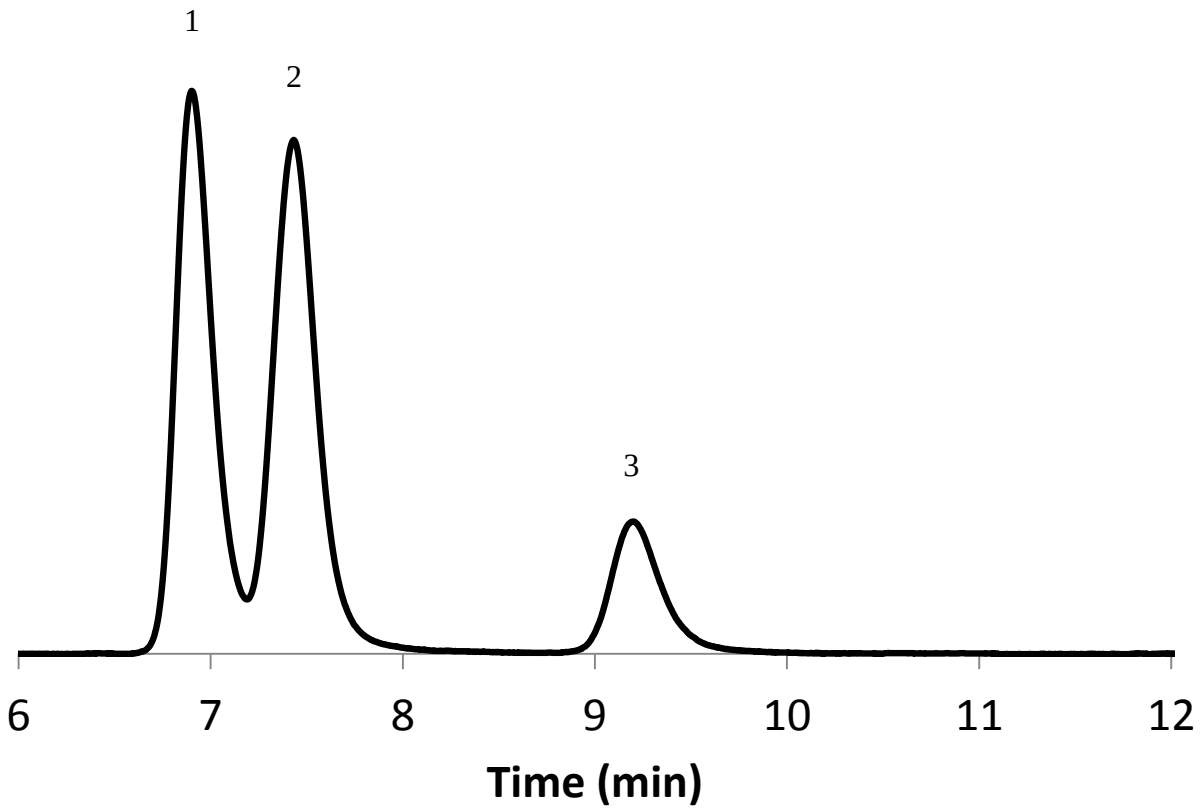

Figure 9. LC condition at a flow rate of 0.4 mL/min. UV detection trace at 262 nm.
1-adenosine 2-cytidine and uridine 3-guanosine

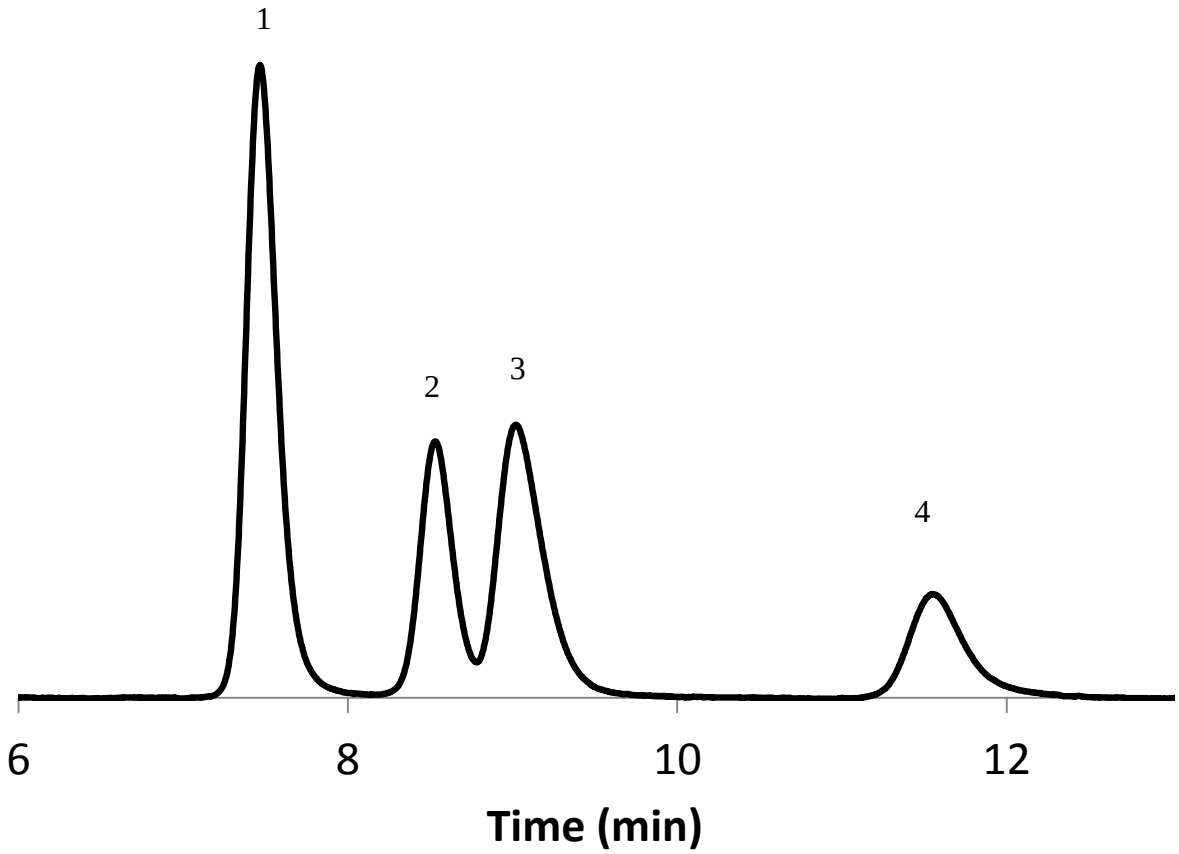

Figure 10. 20 volume percent CO2 added EFL at a flow rate of 0.4 mL/min. UV detection trace at 262 nm. 1-adenosine 2-uridine 3-cytidine 4-guanosine

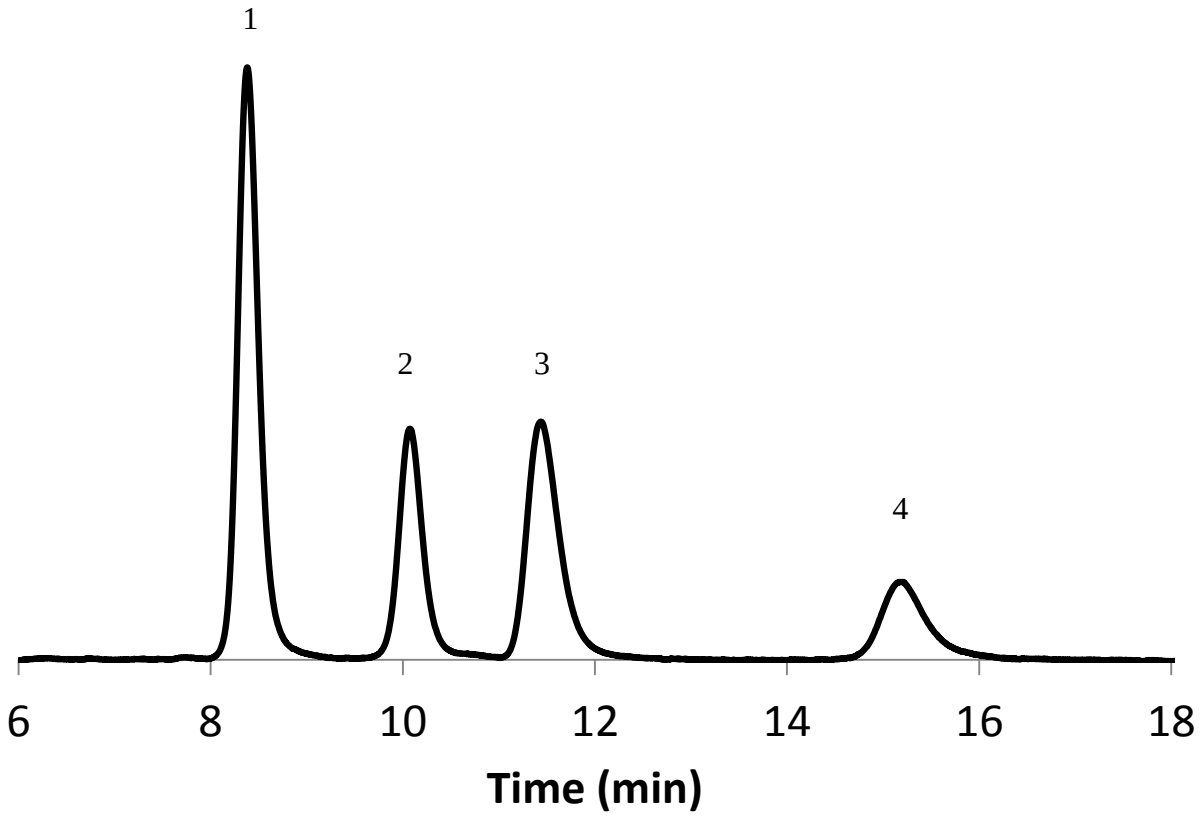

Figure 11. 40 volume percent CO2 added EFL at a flow rate of 0.4 mL/min. UV detection trace at 262 nm. 1-adenosine 2-uridine 3-cytidine 4-guanosine

Nucleoside	EFL f(k)/LC f(k)	Change in f(k)	Experimental ΔH
Adenosine	1.115	+11.5%	-27%
Uridine	1.185	+18.5%	-1%
Guanosine	1.221	+21.1%	+11%
Cytidine	1.263	+26.3%	+17%

Table 1. Comparison of experimental values of ΔH to experimental values of Δf(k).